Scientific Report

Concerning the implementation of the project: **"Functional diversity of D1 Cyanobacterial proteins from photosystem II"** code *PN-II-ID-PCE-2011-3-0765 during October-December 2011*

General considerations concerning the functional diversity of the D1 proteins

Due to strong oxydative reactions involved in the water photolysis process, the D1 protein, unlike other PSII components, it is continously photodegraded and replaced (Aro et al., 1993). The D1 protein is degraded and replaced by a new copy within a span time of 20 minutes to 5 hours in high-light intensity conditions and low-light intensity conditions, in order to maintain an adequate level of the protein necessary to assure a proper functioning of the PSII complexes. It has been observed that at the photosynthetic organisms, the turn-over rate of the D1 protein increases after high-light exposure. Hereby, a severe decrease of this protein quantity can be seen only after a severe stress induced by long time exposure at very high light intensities, leading to the depletion of the photosyntetic activity, photoinhibition (Takahashi & Murata, 2008). As a result, the *psbA* genes expression must be under strict control to guarantee the function of the photosynthetic processes under ever-changing environmental conditions. In higher plants, it is only one psbA gene which encodes the D1 protein, while at cyanobacteria it is present a *psbA* gene family whose members are expressed differentially according to environmental conditions. The presence of multiple *psbA* genes encoding different D1 protein isoforms is an indication of their importance in regulatory mechanisms responsible for maintaining a functional PSII upon changing environmental conditions in natural habitats of cyanobacteria.

In the past two decades, various scientific studies treated the subject of the regulatory mechanisms involved in the expression of cyanobacterial *psbA* genes. Although new data appear continously, it is not known yet a concrete regulatory mechanims, or the transcription factors directly involved. From evidence accumulated so far, the D1 cyanobacterial isoforms can be classified in 4 categories, depending on their expression under typical growth conditions as well as under various environmental stresses (Mulo et al., 2009):

I) $D1_m$, present in PSII reaction centres under normal growth conditions. In stress conditions, the $D1_m$ is induced (eg. $D1_m$ protein codified by *psbA2* ans *psbA3* genes in *Synechocystis* sp. PCC 6803);

II)D1:1, expressed and assembled in PSII under normal growth conditions, repressed in stress conditions (eg. the D1:1 protein encoded by *psbA1* gene in *Synechococcus* sp. PCC 7942);

III) D1:2, a D1 form repressed under normal growth conditions, but induced and accumulated in PSII in stress conditions (eg. the D1:2 protein encoded by *psbA2* and *psbA3* genes in *Synechococcus* sp. PCC 7942);

IV) D1', not expressed in standard conditions, or in usual stress conditions, but induced in microaerobic / low oxygen conditions .

Currently, two types of gene regulation are known at the *psbA* familly members. The first strategy is to replace the D1 protein present in PSII reaction centres under normal conditions (named D1:1) with another protein (named D1:2) encoded by a different gene (or more), in stress conditions. The second strategy, demonstrated in cyanobacteria which have only one type of D1 protein it is to increase, in stress conditions, the turn-over rate of the same protein produced in normal growth conditions (protein named in this case D1m) (Mulo et al., 2009). Recently it has been discovered that at some cyanobacterial species, one of the *psbA* genes (divergent as nucleotidic sequence), known as a „silent gene" until now, it is induced by microaerobic/low oxygen conditions (Sicora et al., 2009; Summerfield et al., 2008). One of these „mysterious" genes, with unknown function exists in 4 out of 5 cyanobacterial species in which the psbA familly gene expression was characterized. The artificial induction of this gene (*psbA1*) at *Synechocystis* sp. PCC 6803 by changing the promoter sequence (Salih & Jansson, 1997), produces a functional protein (D1' protein) (Funk, et al., 2001; Sicora et al., 2004). This gene is present in *Synechocystis* sp. PCC 6803, *Anabaena* sp. 7120 şi *Thermosynechococcus elongatus* BP-1, and it has been demonstrated that at this organisms the

transcript amount increases considerable in microaerobic conditions (obtained by argon bubbling). The evenly answer of this gene at 3 unrelated cianobacterial species, at the same environmental parameter, indicates the fact that this gene and the encoded protein (D1' protein) are part of cellular mechanism of acclimation at microaerobic conditions. There are not ressemblings at structural level between these D1' proteins, besides the 3 aminoacid residues different from the homologous ones from the rest of D1 proteins, which seem to be conserved. These changes in D1' protein sequences are G80 → A, F158 → L şi T286 → A.

The project implementation during October-December 2011

The project started at the 1st of November 2011, when it was hired the project leader and the many of the research team members. Beginning with this date, the activities from the application form have been started or finished as scheduled in the firt part of the project:

- it was initiated the acquisition procedure for the laboratory equipments and the supplies scheduled in this years budget, this budget chapter was used in proportion of 100%.

- it was completed the project research team, as follows: one project leader position, two full-time phD student positions, one full-time master student position and two half-time lab assistant positions involved in the project. It is vacant one post-doc position which will be taken beginning with the third month, according to the financial application and will be announced as vacant on the assigned portal (www.euraxess.ro) and on the other employment media.

- it has been created a data base of DNA sequences and proteins for the D1 protein forms present in the strains whose genomes was sequenced and are available online. These sequences have been aligned, and based on these alignments were created phylogenetic trees which show the relation between the different forms of D1 proteins. 6 cyanobacterial model strains were obtained and their grown optimized in culture. Their genome is sequenced and will be used in the following studies. It has been optimized the ARN isolation protocol, a very important step for the good evolution of the project. A more detailed description and a description of the results it is made in next section.

The establishment of a data base with D1 sequences. In Cyanobase data base, (http://genome.kazusa.or.jp/cyanobase), we found 88 *psbA* genes which encode the different D1 proteins isoforms. The gene sequences have been imported in *CLC Sequence Viewer 6.2* program. Based on the nucleotide sequences we did a multiple alignment of this, followed by a phylogenetic tree in order to measure the evolution distances between the concerning genes. The same procedure was applied for the aminoacid sequences, and the resulting phylogenetic trees are shown in Figure 1.

Optimization of the ARN isolation protocols. In order to optimize the protocols, we tested more ways of breaking the cells using the bead-beater and their effects on ARN isolation at *Synechocystis* PCC6803. On this strain we performed several beat versions, and the experimental results are shown in next table:

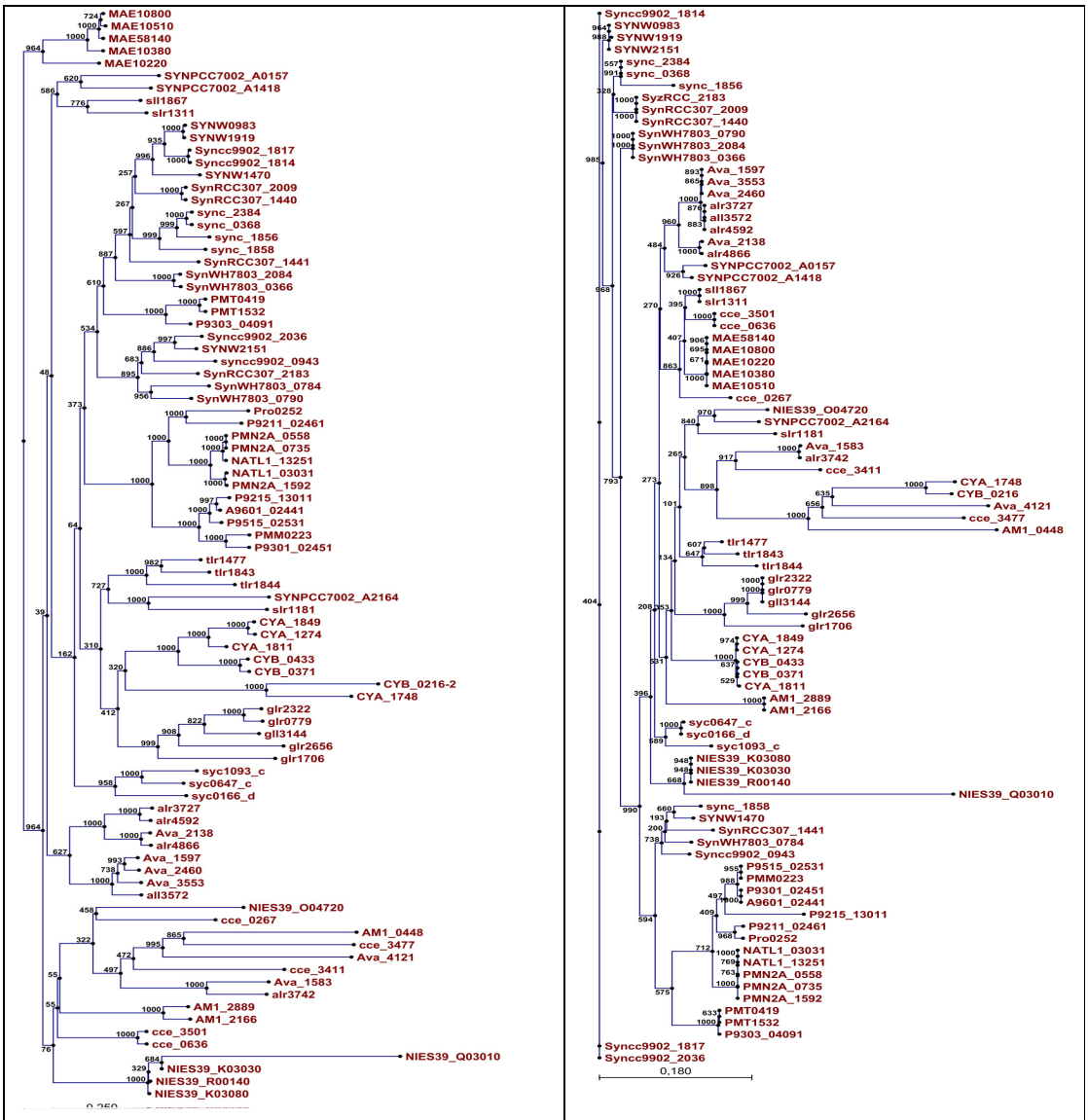
***Synechocystis* PCC6803**	**Version 1**	**Version 2**
ctrl	**588µg\µl**	**504 µg\µl**
cbd	**796 µg\µl**	**449 µg\µl**
cbi	**1150 µg\µl**	**1772 µg\µl**
fbd	**450 µg\µl**	**400 µg\µl**
fbi	**950 µg\µl**	**918 µg\µl**

Legend: Ctrl – control; Cbd – vortex with beads after incubating; Cbi –vortex with beads before incubating; Fbd – vortex without beads after incubating; Fbi –vortex with beads before incubating

From the results shown in table above, it can be seen that shaking the samples with beads before incubation, at maximum speed, for 3-5 minutes, has an effect of considerable increase of isolated ARN amount

Figure 1: Dendrograms showing the relationships between nucleotide sequences (left panel) and the encoded proteins (right panel) of psbA gene families obtained from CyanoBase

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a) phylogenetic inference of nucleotide sequences which encode different D1 protein isoforms	b) phylogenetic inference of aminoacid sequences which encode different D1 protein isoforms

ARN Isolation. To quantify the psbA family gene expression we used an ARN isolation protocol with Trizol, modified after McGinn et all., 2003. One critical first step in this technique is to isolate ARN of high quality and integrity. The steps for ARN isolation are:

1. The process begins with 10 ml cyanobacterial culture, with 60 µg chlorophyll.
2. Collect the cells and add 250 µl Trizol.
3. Incubate 10 minutes at 60° C, after you incubate vortex well on bead beater at maximum power, then repeat the step after 5 minutes and at the end.
4. Add chlorophorm 1/5 volume from the Trizol quantity, wait 5 minutes to cool down before centrifuge it.
5. Centrifuge for 10 min at 10Krpm.
6. Collect the upper phase in an new tube and approximate the volume.
7. Add an equal volume of PCI (phenol\chlorophorm\isoamil alcohol) and vortex well.
8. Centrifuge 2 minutes at 10Krpm.
9. Collect carefully the upper phase and aproximate the volume.
10. Add isopropanol in equal volume and shake well for the phases to mix well.
11. Incubate 10 minutes at room temperature/ covered.
12. Centrifuge at 4° C , 10 minutes at 10Krpm, after this step you can see the ARN pellet.

!!!From this step work only on ice!!

13. Discard the upper phase and add 1ml ethilic alcohol 70%, shake well on vortex.
14. Centrifuge 2minutes/10Krpm/4° C
15. Remove as much ethanol as you can without touching the ARN pellet, centrifuge shortly to remove all the ethanol traces.
16. Let it to dry 30-60 seconds, with tube opened.
17. Add 30µl sterile water. It is important for the pellet to redisolve.
18. Cuantify at spectrometer.

The total ARN amount obtained by using this protocol is of very high quality ARN, and it can be successfull used for downstream applications as cDNA synthesis or in qRT-PCR reactions (data not shown)

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